hands, was active against our tissue culture contaminant, but only when used at considerably higher concentration (50 μg/ml).

It would appear that the concentration of each compound reported to inhibit PPLO strains grown in *broth media* may not be the effective dose against these organisms growing in the *tissue culture* environment, where they are perhaps, protected by their close association with the cells. The advantage of tylan is that it can be used at the high concentration necessary to inhibit PPLO in tissue culture, yet it is non-toxic to the cells.

Summary. A tissue culture line of murine virus-induced leukemia cells was contaminated by a strain of mycoplasma (PPLO) which may be or is related to *M. granularum*. This organism was resistant to treatment with tetracyclin, kanamycin, erythromycin, chloromycetin, polymixin, stovarsol and iodine, but sensitive to Tylosin. Two consecutive treatments with Tylosin (Purina tylan) at a concentration of 50 μg/ml were non-toxic to the cells and were effective in ridding the cultures of PPLO.

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Dissociation of Adult Mouse Liver by Sodium Tetraphenylboron, A Potassium Complexing Agent.* (30951)

CATHERINE RAPPAPORT AND GWENDOLYN B. HOWZE (Introduced by T. T. Puck)
Yale University School of Medicine, New Haven, Conn.

The dispersion of mammalian tissues into a suspension of single cells has usually been effected by the use of enzymes or of the chelating agent, ethylenediamine tetraacetic acid (EDTA). These agents are in general unsatisfactory, since many tissues are refractory to them and even in the case of those which are dissociated, the cells which are liberated are morphologically damaged.

We report here the dissociation of adult mouse liver *in vitro* into a suspension of single cells by sodium tetraphenylboron (TPB). TPB has also been found to dissociate adult mouse brain. Unlike the action of EDTA or trypsin, the cells released retain the gross morphology of the various cell types *in situ*.

It has generally been accepted that cells in tissues are aggregated by some mechanism in which a divalent cation, usually considered to be Ca^{2+}, 'bridges' the negatively charged surfaces of the adjacent cells. TPB is an agent which specifically complexes K^+ (1) and does not complex the divalent cations. The dissociation of tissue by a potassium complexing agent suggests that K^+ is the major cation promoting cell-cell adherence in liver. A coordination mechanism for cellular aggregation is presented and extended to a consideration of the problems of orderly cell movements and of contact inhibition.

Materials and methods. Male C3H mice between 2 and 4 months of age were used. The mice were killed by breaking the neck. The livers were removed and placed in a solution containing .05 M sucrose, 0.14 M of NaCl, and .005 M sodium phosphate buffer, adjusted to pH 7.8. The agents to be tested were added to this solution and when necessary the pH re-adjusted with NaOH.

Tetraphenylboron (Na^+ salt) is obtained from K and K Laboratories, Inc. It is kept frozen as a 1% stock in water and diluted into the dissociation solution just before use. The "sucrose-salt" solution contained .05 M sucrose, .14 M NaCl and .002 M Na_2HPO_4. The pH was adjusted by addition of $NaHCO_3$ as indicated.

Experimental. Dissociation at 4°C. The

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TABLE I. Dissociation of Adult Mouse Liver with Sodium Tetraphenylboron (TPB) at 4°C. Tissue fragments incubated in .05 M sucrose-.14 M NaCl solution at indicated pH for 2 hr and then reduced to suspensions by pipetting. Each number represents an experiment on tissue from a different mouse.

TPB M/L	No. of cells × 10^6 recovered per liver	
	pH 7.8	pH 8.5
None	1.0, 0.8, 1.2, 1.2	2.6, 2.3, 12.0, 14.0
5×10^{-4}	28	
3×10^{-3}	20, 16, 22, 30, 17, 20, 35	65, 70, 81
+ Ca^{2+} and Mg^{2+}*	30	
10^{-2}	38, 40	
3×10^{-2}	46	

* Each at 10^{-3} M.

effect of TPB on dissociation of adult mouse liver tissue is indicated by the data in Table I. In these experiments, freshly excised livers were cut into pieces about 3-5 mm in size. The tissue mass was divided into two or more portions and covered with 10 ml of the sucrose-salt dissociation solutions supplemented or not with TPB at final concentrations ranging from 10^{-5} to 10^{-2} M. The aliquots were kept on cracked ice for 2 hours. At the end of this time the tissue fragments were 'reduced' by pipetting them up and down in a series of pipettes with decreasing bore size. In the absence of TPB the tissue reduces slowly and can be passed through the smaller pipettes only with difficulty after several hours. TPB facilitates the reduction of the tissue mass even at concentrations as low as 10^{-5} M. After incubation in the cold at 10^{-3} M, the tissue can be passed readily through a No. 22 syringe in about 15-30 minutes. The single cells in the fluids were counted by a haemocytometer and the total yield per liver determined by multiplying by the appropriate factor. Tissue from different mice were treated at each of the concentrations. The exact weight of tissue actually processed was not recorded, since a variation of at most 30%, which could be expected from the differences in weight of livers and errors in apportionment of tissue among the solutions, was insignificant compared to the 10-100-fold increases observed in the presence of the complexing agents.

The number of cells released from liver fragments increased with increasing concentrations of TPB (Table I). At 3×10^{-3} M TPB, about 30 times more cells were released than in the sucrose salt controls.

In contrast to the effect of TPB, ethylenediamine tetraacetate (EDTA) at 5×10^{-3} M had no significant effect on dissociation. This agrees with other studies(2) where it was found that even after preliminary perfusion *in vivo*, rat liver was not dissociated by subsequent treatment *in vitro* with EDTA. It indicates that aggregation of cells in liver is not through a divalent cation, since these would be completely complexed at 5×10^{-3} M EDTA at pH 7.8. This conclusion was supported by the finding that addition of Ca^{2+} or Mg^{2+} to the TPB solution did not affect the time required for dissociation or the number of cells recovered.

During the treatment of the tissue with TPB, there is a gradual decrease in pH. Increasing the initial pH of the dissociation solution to pH 8.3 by addition of $NaHCO_3$ in order to compensate for the acid produced, was found to result in as much as 2-fold increase in the number of cells released by TPB (Table I). At the higher pH the number of cells released in the salt-sucrose controls in the absence of TPB was higher and more variable than at pH 7.8. The quality of the cells in these controls was inferior to those released by TPB.

Effect of other agents complexing K^+. TPB is an anion with a high affinity for the potassium ion and also for certain quarternary ammonium ions(1). The finding that it dissociates adult liver tissue into a suspension of single cells indicates that aggregation of the negatively charged cells to each other in the tissue is through some mechanism in which either K^+ or a quarternary ammonium ion plays a major role. In an effort to determine which of the cations is involved in aggregation, the effect of other agents complexing K^+ and also the effect of addition of K^+ and a quarternary ammonium ion, *i.e.*, choline, to the dissociation solutions was investigated.

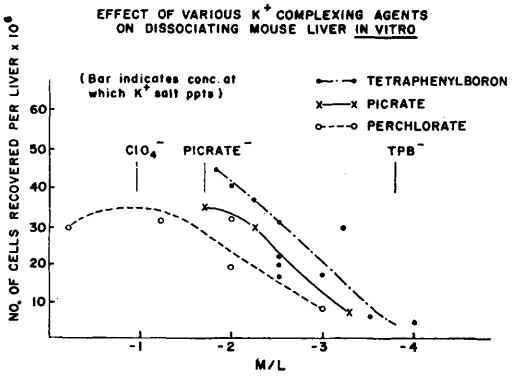

FIG. 1. Number of cells recovered by treatment of adult mouse liver at different concentrations of K^+ complexing agents. Treatment at 4°C in .05 M sucrose-.14 M NaCl solution at pH 7.8.

The anions other than TPB which have been shown to complex K^+ are cobaltinitrite, chloroplatinate, perchlorate and picrate. Cobaltinitrite and chloroplatinic acid were found to lyse cells and their effect on dissociation of tissue could not be evaluated. Sodium perchlorate and sodium picrate were found to dissociate tissue fragments, liberating cells that appeared normal morphologically. The number of cells recovered after treatment in the cold at pH 7.8 as described above at different concentrations of perchlorate and picrate is given in Fig. 1, and compared to TPB. The concentration at which the potassium salts of these anions precipitate has also been given. The correlation between the concentration range optimal for dissociation of the tissue and that at which there is significant association with K^+ as indicated by relative solubilities is particularly good for perchlorate and picrate. The correlation is not good in the case of TPB, which forms an insoluble salt with K^+ at about 2×10^{-4} M, but which shows no significant effect on dissociation until concentrations of 5×10^{-4} M. TPB, however, is a much larger molecule than either picrate or perchlorate and the relative inefficiency may indicate that diffusion into the tissue mass is rate limiting.

The effectiveness of picrate and perchlorate makes it possible to determine by another type of experiment whether dissociation is the result of binding of K^+ or of some quarternary nitrogen compound. In the case of TPB, any K^+ added to the system would precipitate out the TPB, so that even if the effect were due to some other mechanism, there would be an inhibition of dissociation. Potassium picrate is completely soluble at the pH of these experiments, so that the effect of K^+ on picrate dissociation can be investigated. It was found (Table II) that potassium-neutralized picrate was not so efficient as the sodium-neutralized picrate for dissociation. At the same pH and concentration, the sodium-neutralized solution caused the release of nearly 4 times more cells than potassium-neutralized solutions.

Additional evidence that dissociation is the result of the removal of K^+ is indicated by the finding (Table II) that addition of KCl to the sodium picrate inhibited the dissociation whereas addition of choline-Cl, a quarternary ammonium compound, did not.

Morphology of cells released by TPB. Microscopic observation of tissue fragments during treatment with TPB gives additional information on the mode of action of this agent. After only a few minutes in the presence of TPB, the glabrous surface of liver fragments becomes demarcated; gaps form between the cells, and even before many cells are released, it is possible to see the individual cells throughout a fragment. The cells appear to be released by a process, limited to the cell membrane, causing the cells simply to move apart from each other without any change in the shape that they had *in situ* (Fig. 2, 3). Not only polyhedral parenchymal cells, but also endothelial cells with red blood cells still traversing the lumen (Fig. 4), and small cells of the capsule can be seen to separate off intact.

TABLE II. Effect of K^+ on Picrate Dissociation of Adult Mouse Liver.
Dissociation at 4°C in .05 M sucrose-.14 M NaCl at pH 7.8. Each number represents yield of cells from experiments on separate mice.

Supplement	No. of cells × 10^6 per liver
.02 M Picrate (Na^+)	35, 36, 41
+ 10 g/l choline-Cl	39, 31
+ 10 g/l KCl	19
.005 M Picrate (Na^+)	30, 28, 29, 24, 23
.005 M " (K^+)	5, 13
.0005 M " (Na^+)	9
Control—no Picrate	1.0, 0.8, 1.2, 1.2

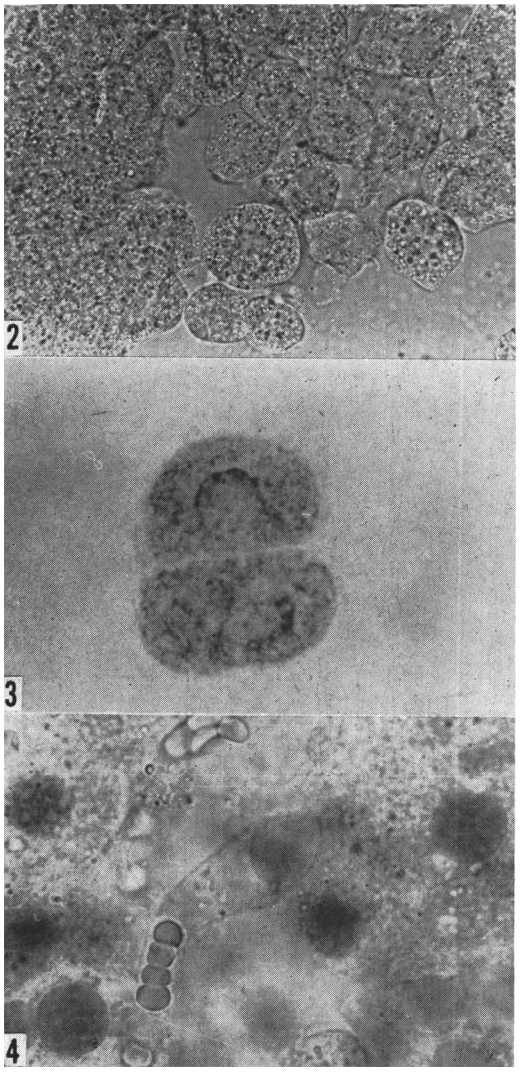

FIG. 2. Separation of parenchymal cells during early stage of dissociation of liver with TPB. Magnification, 104×.

FIG. 3. Two cells separating at cell surfaces during dissociation with TPB. Crystal violet stained. Magnification, 104×.

FIG. 4. Endothelial cell with extended lumen and intracellular red blood corpuscles recovered during treatment with TPB. Magnification, 260×.

A simple experiment indicates the rather extraordinary possibility that virtually every cell in the liver can be recovered as a single intact cell. A fragment taken from the dissociation fluid may be placed on a microscope slide with a cover slip. A very slight pressure of the finger will cause every cell in the fragment to be dispersed. A count of the number of cells in the fragment and the number of cells dispersed by the finger pressure has shown that every cell is recovered separate and intact. This has also been found to occur when the treated fragments are fixed by the usual histological procedures. Fixation and staining of the fragments which had been incubated 15 minutes with TPB show that, without any change in morphology, every cell in the tissue has been separated from contiguous cells. An experiment in collaboration with Dr. Alfred M. Prince, using a biopsy specimen of human liver is shown in Fig. 5.

In the final suspensions prepared by treatment with TPB, many nuclei, as well as single cells were recovered. It would appear that, although separation with TPB can be very efficient, the conditions used here are not optimal for recovery of the separated cells.

TPB was found to have a specific type of toxic effect. Concentrations above 3×10^{-3} M caused a more rapid dispersion of the tissue and a higher yield of single cells. However, many of the cells were devoid of both cytoplasm and nuclei. Only agents complexing K^+, and not any of the agents complexing Na^+ or the divalent cations which have been studied for their effect on dissociation, have been found to produce 'ghosts' in this way. It is possible that even at the lower concentrations, removal of K^+ at surface causes some damage to the cell membrane. However, the single cells recovered

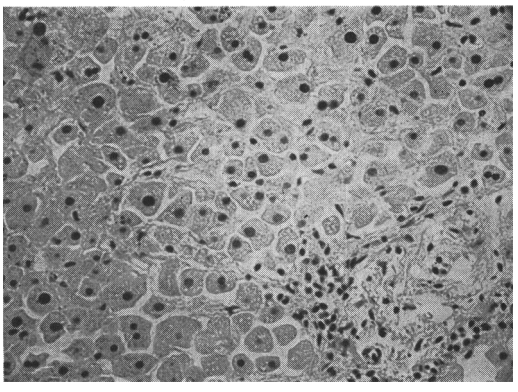

FIG. 5. Fragment of human liver biopsy specimen treated with TPB for 30 minutes. Fixed in 10% neutral formalin and stained with haemotoxylin and eosin. Magnification, 23×.

after treatment with TPB at 3×10^{-3} M are able to repair such damage, since, as will be presented elsewhere, they can grow when inoculated under appropriate conditions.

Discussion. The separation of cells in an adult tissue mass without any change in the gross morphology is the most striking characteristic of the action of sodium tetraphenylboron (TPB). Not only polyhedral parenchymal cells, but endothelial cells with extended lumen can be seen to separate off. The recovery of cells with such delicate morphology indicates that the action of TPB is primarily on some mechanism restricted to the cell surface. The cells are in good physiological condition, and have been found to grow *in vitro* when inoculated under suitable conditions.

TPB complexes K^+ and certain quarternary ammonium compounds, but does not complex Na^+ or the di- or tri-valent cations (1). Dissociation with TPB thus suggests that either K^+ or some quarternary nitrogen—possibly that which is a normal component of the cell membrane—is in some way neutralizing the negative charges on the surfaces of the cells. The effect would appear to be the result of the removal of K^+ rather than to a binding of the quarternary nitrogen residue, since two other K^+ complexing agents, *i.e.*, picrate and perchlorate, also separated the cells. Furthermore, it was found that the K^+-neutralized picrate was less effective than the sodium-neutralized and that dissociation with the sodium salt was inhibited by excess KCl, but not by choline-Cl, a quarternary ammonium compound.

When considering the mechanism by which K^+ can cause the aggregation of cells, it is not necessary at this time to distinguish between the case in which cells adhere directly to each other, and that in which aggregation involves some intercellular matrix material. Macromolecules of intercellular materials, as well as cells, have been found to carry a net negative charge at physiological pH's. The problem in both cases is the aggregation of two mutually negatively charged surfaces. Any model proposed, however, must attempt to explain the two important characteristics of aggregation in tissues, *i.e.*, selectivity of cells aggregating and maintenance of specific aggregates under conditions of electrolyte exchange.

Previous theories of aggregation have all proposed that a divalent cation—presumably Ca^{2+}—in some way bridges the two negative surfaces. The early finding that removing Ca^{2+} either by Ca^{2+}-deficient salt solutions or by adding the complexing agent, ethylenediamine tetraacetic acid (EDTA), resulted in dispersion of some tissues into single cells was the major experimental evidence in favor of this theory. However, only certain embryonic and invertebrate tissues are dissociated effectively by these treatments (see Mascona(4) for reviews). Adult mammalian tissues have, in general, been found to be refractory to removal of Ca^{2+}.

The finding that an adult mammalian tissue is completely dissociated by an agent which specifically complexes K^+ is not consistent with the hypothesis that Ca^{2+} is the major cation promoting aggregation. It suggests instead a mechanism of aggregation by coordination of the negative surfaces about monovalent cations. In liver, the major cation would appear to be K^+, but a general theory would have to allow for coordination about Na^+. It may be noted that since EDTA complexes Na^+(4,5) as well as Ca^{2+}, this hypothesis is consistent with some of the earlier findings. We have used this model for a series of studies, including both studies on dissociation of tissue, and on attachment of cells to glass. These studies will be presented in the following papers. Presented here are some of the aspects of coordination chemistry which may be usefully considered in analyzing the problem of specific aggregation and the related problem of movement of cells in tissues.

The aggregation of cells in tissues may be considered analogous to the structure of certain minerals. In mice, for example, negatively charged layers are held together by coordination about K^+. In still others, the cation of aggregation is Na^+. Whether the layers form stable aggregates about Na^+ or K^+ is determined at single cation loci in the layers.

The structure of the aggregate is also determined by the number of anionic sites, *i.e.*, coordination number, on the layers which by

surrounding the cation shield its positive field.

Layers will adhere to each other only if their anionic sites can occupy coordination positions about the intervening cation. Likewise, in mammalian systems, one cell will adhere to another cell only if the chemistry of their surfaces is such as to satisfy the coordination requirements of the surface bound cation. A difference in the chemistry of Na^+ and K^+ in mineral structures may be an important feature of the model for cellular interactions. In the case of Na^+ only one configuration, in which there are 6 anions symmetrically oriented in space, is commonly found to occur. K^+, however, forms several types of complexes differing both in number of anions and their spatial arrangement(6). This may suggest that K^+ will be the more general cation of aggregation in tissues, since it would allow for a certain number of selective interactions among cells.

It is also proposed that, unlike minerals, where sites on the layers are seldom replaced by soluble anions, *i.e.*, 'ligands,' in the cellular system a certain number of the sites can be replaced by mobile ligands. Many of the common electrolytes coordinate with Na^+ and K^+, *e.g.*, H_2O, Cl^-, OH^-, etc. Thus, electrolyte exchange takes place during aggregation as the result of interchanges within the cations coordination sphere of fixed sites on the cell surface by mobile ligands. Aggregation is maintained as long as any 2 of the available coordination positions are occupied, one cell each, by sites on the cell membranes. In this model, the configurations permissible at the sites of aggregation for a given cell, are determined by the chemistry of the surface, which determines both the number and type of ligands acceptable. The actual complex realized, however, would depend on metabolism. In this way, aggregation and ordered movements of cells become coupled with electrolyte exchange.

According to this model, a cell can be characterized as having a set of 'permitted' configurations in which it is stable *vis-a-vis* another surface. A different cell type by virtue of a different surface structure would have a different set. This distinction is sufficient to account for ordered movements of cells in tissues. A cell proceeds through the tissue as the result of contacts with other cells. This is determined at any given moment by whether the sites on the surface of the approached cell can complete coordination about the surface-bound cation of the contacting cell. This means that the two cells must have at least one of their permitted configurations in common and that this mutually permitted configuration can be realized under the metabolic conditions prevailing at the moment of contact. The contact between cells which would have only one mutually permitted configuration would be the most unstable and would have limited duration, since the cells would tend to proceed toward a more stable configuration. The most stable complex would be the one in which coordination was completed by two identical sets of 'fixed' anionic sites, *i.e.*, when the cells were identical. The stability increase occurring in complexes where the same ligand occupies more than one coordination position about a cation would be, in the case of complexes between cells, further amplified by cooperative effects on the cell membranes. Thus cells would sort out by 'contact guidance' according to cell types. The stability afforded by coordination of 'identical' surfaces would be the biological equivalent of 'recognition.' It should result in decreased movements of cells or 'contact inhibition.' It should also involve a shift in the electrolyte metabolism of the cells.

The model for aggregation which has been proposed is a direct application of coordination chemistry following the observation that cells in liver appear to be aggregated about K^+. It has attempted to explain the two biologically significant aspects of aggregation, *i.e.*, selectivities exhibited by cells in aggregates and stability of aggregates under conditions of electrolyte flux. Furthermore, it states explicitly that dynamics of cells in tissues is coupled with electrolyte exchange. Investigation of some of the questions specifically raised by this model may elucidate the exact nature of this coupling and its consequences on metabolism and growth.

Summary. 1. Sodium tetraphenylboron (TPB), a specific agent for complexing K^+ has been found to dissociate adult mouse liver

in vitro into a suspension of single cells. 2. Evidence is presented that this is the result of the removal of K^+, which is the major cation involved in aggregation of cells in this tissue. 3. A coordination mechanism for aggregation is proposed in which the negatively charged surfaces in cell-cell aggregates or in cell-matrix-cell aggregates are neutralized by monovalent cations. 4. Two variables of coordination mechanisms, *i.e.*, cation coordinated and the coordination number are used in a model advanced to explain ordered movement and aggregation of cells in tissues.

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Further Studies on the Dissociation of Adult Mouse Tissue.* (30952)

CATHERINE RAPPAPORT AND GWENDOLYN B. HOWZE (Introduced by T. T. Puck)
(With the technical assistance of Mehda von Essen and Joan E. Marino)
Yale University School of Medicine, New Haven, Conn.

The dissociation of the adult mouse liver by sodium tetraphenylboron (TPB) has been reported(1). The selectivity of TPB for the K^+ ion(2) and the failure of EDTA(1,3), an agent complexing Na^+(3,4) and divalent cations suggested that the cells were held together in the tissue by coordination through K^+. This rather unexpected finding has been investigated further by studying the effect of other agents complexing cations on the dissociation of liver. Dissociation of other adult tissues, *i.e.*, kidney, brain and connective tissue, was also investigated in order to see if coordination through monovalent cations were a general mechanism for aggregation of cells in tissues.

The results indicate that K^+ is the major cation involved in aggregation in many adult mouse tissues. Certain differences have been found in the optimal agent for recovering particular types of cells from different tissues. The results indicate that coordination through monovalent cations is a general mechanism for aggregation, but that tissues may differ in the number of sites on the surface occupied by Na^+ or by K^+, and possibly also by the degree of hydration of the surface bound cations.

Materials and methods. Adult C3H male mice from 2-4 months old were used. The animals were killed by breaking the neck. The tissues were removed immediately and placed into the dissociation solution and allowed to stand at 4°C. The dissociation solution for liver contained 0.05 M sucrose, 0.14 M NaCl and .005 M sodium phosphate buffer pH 7.8. The complexing agents were diluted directly into this mixture from concentrated stocks and when necessary the pH was adjusted with NaOH.

In experiments with kidney, brain and connective tissue, the concentration of the sucrose was also varied.

All organic reagents were obtained from the K. and K. Special Chemical Laboratories. Sodium metaborate was obtained from Fisher Scientific Co.

Comparison of Na^+ and K^+ complexing agents. The agents which have been studied with respect to their capacity to complex Na^+ or K^+, as determined by a survey of the

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